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Mitochondrial Activities of Phosphagen Kinases are Not Widely Distributed in the Invertebrates

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A diverse array of phosphagen kinases [arginine kinase (AK), lombricine kinase (LK)], glycocyamine kinase (GK), taurocyamine kinase (TK), and creatine kinase (CK) is found in the animal kingdom (see ref. 1 for a review). These reactions appear to function in the temporal buffering of ATP in muscles during energy deficits such as might occur during burst contraction or anoxia (2, 3). In many vertebrate tissues, a distinct mitochondrial isoenzyme of CK is present, and it may play a special role in the intracellular transport of high energy phosphate (4). In this study, we investigated whether mitochondrial activities of phosphagen kinases are present in invertebrate muscles. Our results show that AK is present in mitochondria from a crustacean. However, phosphagen kinases are lacking in mitochondria from insect flight muscles, molluscan cardiac and smooth muscle, and polychaete and oligochaete body wall musculature. It appears that mitochondrial activities of phosphagen kinases are not widely distributed in the invertebrates. These data, in conjunction with previous studies on the physico-chemical nature of the interaction of phosphagen kinases with mitochondria (5, 6), suggest that mitochondrial compartmentation of phosphagen kinases may have evolved independently in two major animal groups.

Mitochondrial CK in vertebrate muscle constitutes the proximal end of the so-called phosphocreatine shuttle (4). According to the shuttle model, CK catalyzes the phosphorylation of creatine to phosphocreatine using newly synthesized ATP. The resulting phosphocreatine is then thought to diffuse from the mitochondrion to sites of ATP use (myofibrils, ion transport ATPases), where it is used to phosphorylate ADP to ATP. In effect, high energy phosphate is thought to be transported by phosphocreatine rather than ATP, which overcomes the diffusion limitations of the adenine nucleotides (4). The presence of mitochondrial CK is advantageous because it maximizes enzymatic potential in the compartment where it is needed (2). In contrast to the situation in vertebrate muscles, we show in the following results that mitochondrial activities of other phosphagen kinases are rather uncommon in the animal kingdom.

Tightly coupled mitochondria were isolated from the muscles of seven representative species of invertebrates, and the presence of phosphagen kinase activity was assessed by respirometric methods (Table I). Phosphagen kinase activities were not present in mitochondria from the body wall musculature of the earthworm *Lumbricus terrestris* and the polychaete *Nereis virens*. Mitochondria from the radula retractor muscle of the whelk *Busycon canaliculatum* and the systemic ventricle of the octopus *Octopus vulgaris* lacked AK activity, which is consistent with results from studies on other mollusks (8, 9, 10). AK was also not present in mitochondria from the flight muscles of the blowfly *Sarcophaga bullata* and moth *Manduca sexta*. AK also appears to be absent from the flight muscle mitochondria of the locust *Locusta migratoria* (11). Spectrophotometric assays (3) of phosphagen kinase activities in detergent extracts of *L. terrestris*, *N. virens*, and *M. sexta* mitochondria revealed only trace (<0.1 μmole/min · g wet wgt at 25°C), or no activity.

Only mitochondria isolated from the hearts of the crayfish *Procambarus clarkii* contained phosphagen kinase activity (Table I). Mitochondrial AK activity in *P. clarkii* was sufficiently high, as to facilitate stimulation of approximately 50% of state-3 respiration when 5 m*M* L-arginine was added to the respiration system (Fig. 1). Mitochondrial AK represented around 1.5% of the total AK

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Table I

Mitochondrial activities of phosphagen kinases in the muscles of a variety of invertebrates. A "+" or "−" indicates the presence or absence, respectively, of mitochondrial kinase activity. Quality of mitochondrial preparations is indicated by showing the range of values of the respiratory control ratios (RCR = State 3 respiration ÷ State 4 respiration). An RCR value greater than one indicates that mitochondria are coupled and show respiratory control behavior in response to the addition of ADP (see discussion below)

Organism and tissue	RCR	Phosphagen kinase	Mitochondrial activity
Lumbricus terrestris body wall (earthworm)	3–4	LK	−
Nereis virens body wall (polychaete)	4–5	GK	−
Busycon canaliculatum radula muscle (whelk)	3–5	AK	−
Octopus vulgaris systemic ventricle (octopus)	8–17	AK	−
Sarcophaga bullata flight muscle (blowfly)	4–7	AK	−
Manduca sexta flight muscle (moth)	5–10	AK	−
Procambarus clarkii heart (crayfish)	4–6	AK	+

N. virens and *B. canaliculatum* were obtained from the Marine Biological Laboratory (Woods Hole, Massachusetts). *L. terrestris*, *S. bullata*, and *M. sexta* were purchased from Carolina Biological Supply (Burlington, North Carolina). *O. vulgaris* and *P. clarkii* were collected locally. Mitochondria were isolated by gentle homogenization and differential centrifugation procedures (details available upon request). Mitochondrial respiration was monitored polarigraphically as previously described (6, 7). The addition of ADP to tightly coupled mitochondria respiring in the presence of substrate (state-4) leads to a dramatic increase in respiration (state-3) which will continue until all of the ADP has been phosphorylated to ATP. If a phosphagen kinase is present in the mitochondria, subsequent addition of the appropriate phosphagen acceptor (arginine, lombricine, glycocyamine, etc.) will lead to the formation of phosphagen and ADP by the following reaction:

Phosphagen kinase	Acceptor + ATP → ADP + Phosphagen
Oxidative phosphorylation	βO_2 + ADP + P_i → ATP + βH_2O
Net reaction	βO_2 + Acceptor + P_i → Phosphagen + βH_2O

(Note: β is dependent on the P:O ratio)

The resulting ADP will stimulate respiration and ATP formation via oxidative phosphorylation (see above). The ATP will phosphorylate additional acceptor, producing more ADP which will stimulate state-3 respiration as long as acceptor is present (net reaction above). Thus, stimulation of state-3 respiration by phosphagen acceptor indicates the presence of mitochondrial phosphagen kinase activity (see Fig. 1, for example), providing that the mitochondria have been extensively washed, as was the case in this study. Most experiments were conducted on at least three independent preparations from each species. Because *O. vulgaris* was not readily available, only a single mitochondrion preparation was used for the experiments with this species.

activity in *P. clarkii* heart muscle (Fig. 1). AK has also been observed in the mitochondria of several other crustaceans (12, 13, 14). Furthermore, we have recently shown that heart mitochondria from the horseshoe crab *Limulus polyphemus* (a chelicerate arthropod) contain AK activity that is clearly intrinsic to the mitochondrion (6, 7).

Our rather limited survey, coupled with the results of others, suggests that mitochondrial phosphagen kinase activities are consistently present in the muscles of only three groups: AK in crustaceans, and also in the relic chelicerate *L. polyphemus*, and CK in vertebrates. The interaction between AK and these mitochondria is hydrophobic, in that detergents are required to solubilize enzyme activity (6, 14). In contrast, the interaction between CK and vertebrate mitochondria is clearly electrostatic and is easily disrupted by changes in ionic strength

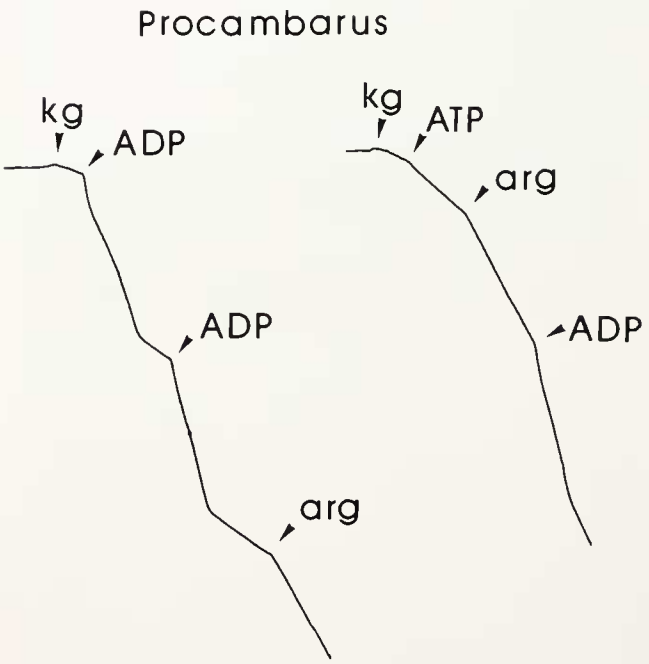

Figure 1. *Patterns of oxygen consumption (vertical-oxygen concentration; horizontal-time) of mitochondria from the heart of the crayfish* Procambarus clarkii. *Mitochondria were added to an isotonic respiration medium supplemented with 1.5 mM $MgCl_2$ and 5 mM potassium phosphate, and respiration was monitored at 25°C.* Left panel—*5 mM α-ketoglutarate (Kg) was initially added followed by two cycles of addition of 200 μM ADP. After return to state-4 respiration, 5 mM L-arginine (arg) was added to ascertain whether AK was present.* Right panel—*Respiration was initiated by the addition of 5 mM α-ketoglutarate followed by 200 μM ATP. Addition of 5 mM L-arginine resulted in stimulation of State-3 respiration via ADP production by the AK reaction. Respiration was further enhanced by addition of 200 μM ADP. In both sets of experiments, L-arginine was capable of stimulating respiratory activity equivalent to approximately 50% of the ADP-initiated state-3 rate. To verify the presence of AK activity,* P. clarkii *mitochondria were extracted in detergent (1% Triton X-100). AK activity was assayed in the mitochondrial extract using previously described spectrophotometric procedures (7). AK activity in crayfish mitochondria was 16 μmoles/min·g wet wgt at 25°C which represents approximately 1.5% of the total AK activity in this tissue. Since these mitochondria were washed four times, it is clear that AK activity is intrinsic to* P. clarkii *mitochondria and is not a cytoplasmic contaminant.*

(5). Given the broad phylogenetic distance between the crustacean and chelicerate arthropods and the vertebrates, the apparent lack of phosphagen kinase activities in the muscle mitochondria of the other major groups, and the dramatic differences in the physico-chemical interaction between these kinases and the mitochondria, we speculate that mitochondrial phosphagen kinase activities arose independently in the two groups where they are found.

Finally, we point out that, although insect flight muscles and cephalopod hearts develop the highest aerobic power outputs of any invertebrate muscles (15, 16), the functional capabilities of these muscles do not appear to be intrinsically limited or compromised by the absence of phosphagen kinase activities in their mitochondria.

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